3 1151 02563 4993

MORAVIA
SPEC. COLL.

JHU THESIS
Parsons,
Elizabeth I.
1926
C. 1

THE JOHNS HOPKINS UNIVERSITY
SCHOOL OF HYGIENE AND PUBLIC HEALTH
310-312 W. MONUMENT STREET
BALTIMORE, MD.

DR. PARSONS' THESIS

PUBLISHED

JOURNAL OF IMMUNOLOGY

VOL. XII, No. 1, July 1926, p. 47-82

STUDIES IN COMPLEMENT SPLITTING WITH SPECIAL REFERENCE TO THE ACTIVATION OF YEAST ABSORBED AND COMPLEMENT DEFICIENT GUINEA PIG SERUM

DISSERTATION
Submitted to the Advisory Board of the School of Hygiene and Public Health of the Johns Hopkins University in Conformity with the Requirements for the Degree of Doctor of Science in Hygiene

Elizabeth I. Parsons,
Baltimore, Maryland
May 1, 1925

CONTENTS

	Page
Introduction	1
Experimental	4
Splitting of Guinea Pig Complement into Two Pieces	4
Splitting of Guinea Pig Complement into Three Pieces	19
Splitting of Guinea Pig Complement into Four Pieces	30
Reactivation of Yeast Absorbed Guinea Pig Complement	33
Activation of Deficient Guinea Pig Serum	42
Discussion	52
Summary	55
Bibliography	57
Biography	62

STUDIES IN COMPLEMENT SPLITTING WITH SPECIAL REFERENCE TO THE ACTIVATION OF YEAST ABSORBED AND COMPLEMENT DEFICIENT GUINEA PIG SERUM

INTRODUCTION

In 1907 Ferrata (11) demonstrated that guinea pig serum which had been freed from salts by dialysis against running water was not effective in hemolytic reactions. He further demonstrated that the serum fractions, obtained by centrifuging the precipitate of globulins from the fluid or albumins of the serum, were inactive in themselves. The complementing activity, however, was restored when the two were mixed, for the mixture was potent in hemolytic reactions. He attributed his results to the "splitting of complement" during the process of dialysis, for he was able to show that the amboceptor became bound to the red cells during the reaction. Sachs and Teruuchi (32) in the same year, had failed to obtain hemolysis with complement which had been diluted with distilled water. They, however, explained their results in terms of an enzyme which they assumed to be active only in solutions of low salt concentration.

Other workers immediately siezed upon the work of Ferrata in an attempt to determine the constitution of complement. Brand (2) as well as Hecker (15) confirmed his work for the most part and added certain new facts. Brand was able to show that the globulin portion of the serum became bound to the sensitized cells before the albumin portion. Following Ehrlich's conception he called the globulin fraction the "midpiece" and the albumin fraction the "endpiece" of complement. That is, the globulin and albumin fractions of complement stand in the same relation to amboceptor as do amboceptor and complement to the antigen. This terminology has met with ill defined objections from some workers, but since it is used throughout all the literature and no other has been suggested to take its place, it will be used in this paper.

Several investigators who followed Ferrata and Brand found that dialysis was for many reasons an unsatisfactory method of splitting complement. Other methods were then devised, the most important of which were the Sachs and Altmann HCl Method (30) and the Liefmann CO_2 Method (22). All the methods except the latter split the complement so that the midpiece contains the globulins of the serum and the endpiece the albumins, when the separation is complete. The Liefmann method produces a mid-

piece which contains the euglobulins and a small portion of the pseudoglobulins, and an endpiece which contains the bulk of the pseudoglobulins and the albumins of the serum, due to the property possessed by the CO_2 gas of splitting the pseudoglobulins. In 1914 Browning and Mackie (5) carried the work further and reported methods of splitting complement into three and four pieces. In this work they made use of the fact that it is possible to salt out the various proteins of the serum by means of a saturated solution of $(NH_4)_2SO_4$.

The many problems presented by the possibility of splitting the complement have been the object of careful experiment by many different workers, although if one examines the literature it will be seen that almost no work has been done in this field during the past ten years. As a result of the vast amount of research done from fifteen to twenty years ago, and the contradictory results obtained, the whole field has been left in a state of great confusion. The aim of the present work has been to review and repeat the work of the most important of these workers and to re-evaluate their claims. In light of these claims we propose to present certain new facts in connection with guinea pig complement which has been rendered inactive with yeast, and with fresh guinea pig serum from the complement deficient guinea pig, an animal in

which a component of complement is naturally missing.

EXPERIMENTAL

In all of the following experiments the guinea pig complement was collected from several pigs by cardiac puncture and pooled, and the blood allowed to clot. The clot was broken up very thoroughly and the serum centrifuged off immediately. The sensitizer was obtained by cardiac puncture from rabbits that had been injected with washed sheep cells. This sensitizer was inactivated at 56° C. for 30 minutes. Several such sensitizers of varying titres were used during the course of the experiments, 5 units of each in 0.1 cc. dose being the amount employed. The sheep cells were collected in citrate and washed in large amounts of isotonic salt solution four times. The unit of cells used in all experiments was 0.1 cc. of a 1 in 4 dilution in terms of whole blood. All readings were made immediately after incubation and in terms of complete hemolysis.

Splitting of Guinea Pig Complement into Two Pieces

Liefmann Method (22): A given amount of complement is diluted with four volumes of distilled water, making a 1 in 5 dilution of the original serum. A few drops of capryl alcohol are added to prevent the foaming of the

serum when the CO_2 gas is passed through. The gas, from a tank under pressure, is passed through the diluted serum from 15 to 20 minutes at moderate speed. This time is sufficient for as complete a precipitation of the globulins as possible. The precipitate thus formed is immediately separated by centrifuging and the clear supernatant liquid, which retains the original color of the serum, poured off through a hardened filter paper to remove all traces of the precipitate. This supernatant liquid, or endpiece of complement, is made isotonic by the addition of the required amount of sodium chloride crystals. The precipitate, or midpiece of complement, is immediately washed three times with large amounts of distilled water, and stirred up thoroughly each time before centrifuging. The midpiece is then dissolved in isotonic sodium chloride in an amount equal to that of the original diluted serum. Mid piece and endpiece are therefore both obtained in dilutions of 1 in 5. The midpiece dissolves only after 15 to 20 minutes. Despite the fact that it is stirred frequently the solution remains somewhat cloudy.

Kiss Method (20): A modification of the Liefmann CO_2 method, which was sometimes used, is that described by Kiss. Instead of diluting the serum with distilled water it is diluted with distilled water that has been saturated with CO_2 gas. This process is believed

to cause less injury to the complement. The precipitate formed is centrifuged off immediately after dilution with the water saturated with CO_2. We find that when the complement is subjected to this method the precipitation is not always complete and an endpiece is frequently obtained which is hemolytic in itself.

Modification of the Two Methods: Since the dilution with distilled water has been shown by some workers to have a harmful effect on complement, and since the Kiss method in itself is not entirely satisfactory, the following modification was used in many experiments. After adding the water saturated with CO_2 the gas is allowed to pass through the solution for from five to ten minutes in order to insure complete precipitation. The remainder of the procedure is identical with that described under the Liefmann method. The CO_2 gas has the property of splitting the pseudoglobulins of the serum, and therefore the midpiece contains the euglobulins and a small portion of the pseudoglobulins, while the endpiece contains the bulk of the pseudoglobulins and the albumins.

Both the Liefmann and the modified methods were used in carrying out our experiments and there was practically no difference in the end results obtained. Under no circumstances was an endpiece or a midpiece obtained

which was hemolytic in itself in a 1 in 5 dilution.

Experimental: Protocol I, a and b, are those used in determining the hemolytic activity of the two pieces when mixed. These were allowed to stand together ten minutes or more before setting up the protocols.

Protocol I

Type used in determining the activity of complement after splitting with CO_2.

a	Tubes	1	2	3	4	5	6	7	8
	Sensitizer 5 Units	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	+ Midpiece(1-5) Endpiece(1-5)	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
	Salt Solution	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7
		Incubate 20 minutes at 37°C.							
	Sheep Cells(1-4)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
		Incubate 60 minutes at 37°C.							
b	Tubes	1	2	3	4	5	6	7	8
	Sensitizer 5 Units	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Sheep Cells(1-4)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Salt Solution	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7
		Incubate 20 minutes at 37°C.							
	+ Midpiece(1-5) Endpiece)1-5)	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
		Incubate 60 minutes at 37°C.							

Table I shows the results of twelve experiments taken at random from the many made.

Table I

This table shows the titre of complement before and after splitting with CO_2.

No.	Titre of Complement before Splitting	Titre of Complement after Splitting	
		Cells Added Last	Sensitized Cells
1	1-200	1- 60	1- 60
2	1-200	1- 50	1- 50
3	1-200	1- 60	1- 75
4	1-200	1-100	1-100
5	1-135	1- 35	1- 40
6	1-125	1- 50	1- 50
7	1- 80	1- 50	1- 40
8	1-160	1- 30	1- 33
9	1-115	1- 50	1- 45
10	1-115	1- 50	1- 25
11	1-200	1- 50	1- 60
12	1-110	1- 50	1- 50

The above table shows that there is a decided loss in the activity of complement when it is split with CO_2. The average titre of the complement before splitting

in these twelve cases is 1-155. The average titre after splitting when the cells are added last is 1-53, while when sensitized cells are used it is 1-52. The complement has therefore lost about two-thirds of its activity. Braun (3) stated that in his work on the splitting of guinea pig complement with CO_2 the loss of activity was from one-third to one-half. In our experiments the loss of complement activity was never as low as one-third.

It is seen from table I that it makes very little difference in the end results whether the cells are added last or sensitized cells are used. Slight differences occur but they may be in one direction or the other without any seeming regularity. The table shows no correlation between the original titre of the serum and the titre after fractioning.

Brand's Modification: In determining the exact conditions under which hemolysis takes place when midpiece and endpiece are mixed, the following observations were made:

If the endpiece is left in contact with the centrifuged midpiece as long as two and one-half hours, no hemolysis takes place. If the midpiece stands that length of time without washing no hemolysis follows, although it is necessary to wash it but once in order to obtain hemolysis. Our experiments have indicated that

this is due to a change in the midpiece and not in the endpiece. If the midpiece remains in isotonic salt solution for some time it does not act when mixed with the endpiece. This loss of activity which the midpiece undergoes when standing in isotonic sodium chloride is called "Brand's modification". That it is a modification of the piece and not a deterioration, is shown by the fact that if the midpiece is first added to the sensitized cells and then the endpiece, after a delay of from five to ten minutes, hemolysis will take place for some time after it is impossible to obtain it when midpiece and endpiece are mixed. This has been called by Michaelis and Skwirsky (26) "persensitization" of the cells. The negative results obtained under the conditions stated above are probably due to a modification of the midpiece.

The change which takes place in the midpiece of complement after standing in isotonic salt solution was first noted by Brand in 1907, and since that time a large amount of work has been done on this point. Marks (24) examined the sera from twenty guinea pigs and found that modification took place in all within twenty-four hours. We observed one serum which remained active for thirty hours, while we had others which became changed in an

hour or two. Ledingham and Dean (21) reported that if the fractioning were carried out at 0°C. and the pieces kept at that temperature, the midpiece remained active for weeks and the endpiece for months. Our experiments showed that modification took place more quickly when the fractioning was made at 37°C. than at room temperature or that of melting ice. Also more quickly when water saturated with CO_2 was used than when the dilution was made with distilled water.

Many theories have been advanced as to the cause of this modification. Brand himself thought that it was due to a deterioration of the midpiece, but this has been disproved by the hemolysis resulting with persensitized calls. The Ehrlich school, represented by Hecker and others, explained it in terms of a change in combining affinities. The midpiece loses its affinity for the sensitized cells and gains in affinity for the endpiece. Ledingham and Dean explained the phenomenon as due to a change in the aggregation of the particles of globulin in suspension. Kiss maintains that the so-called modification is nothing but the loss of activity of the complement present in the midpiece. After this has taken place the solution contains only an inhibiting substance which he calls anticomplement. Thomson and

Leschly (34) put forward what seems to be the most likely explanation. According to them the modification is due to a restricting substance present in the serum, probably the euglobulins. They base their conclusions on the following facts: (a) The globulin when modified inhibits the action of normal complement. (b) Large doses of the modified globulin are not active in combination with endpiece, but small doses are. (c) Friedemann (13) showed that it was possible to remove midpiece and have the inhibiting substance remain in solution.

Variations in the Conditions of Complement Splitting: We varied the conditions under which the fractioning was done. There seemed to be no difference in the amount of hemolysis obtained when the fractioning was carried out at 37°C. or at the temperature of melting ice, but the amount of precipitate formed at 37°C. was somewhat larger. If the serum was diluted with isotonic sodium chloride instead of with distilled water, the anount of precipitate which formed was small and the endpiece was hemolytic in itself. The addition of the salt evidently prevented the globulins from precipitating out of solution. If the midpiece was washed with distilled water saturated with CO_2 instead of with plain distilled water, some of the precipitate seemed to dissolve out.

This washing with CO_2 water inhibited the resulting hemolysis to some extent, but did not prevent it, even though the midpiece stood after washing as long as two hours before being dissolved in isotonic sodium chloride.

Kiss Method: We have carried out a number of experiments using the Kiss method unmodified. The results are shown in table II.

Table II

This table shows the titre of complement before and after splitting with the Kiss Method, and also the hemolytic activity of the pieces.

Number	Titre of Complement before Splitting	Titre of Complement after Splitting		Hemolytic Activity	
		Cells Added Last	Sensitized Cells	Midpiece	Endpiece
1	1-300	1-200	1-150	1-4	1-8
2	1-250	1-125	1-150	0	1-4
3	1-200	1-100	1- 85	0	1-3
4	1-175	1-175	1-125	0	0
5	1-135	1-100	1- 50	0	1-2
6	1-120	1- 50	1- 50	0	0
7	1-130	1- 40	1- 45	0	1-4
8	1-135	1-100	1- 60	0	0

It will be seen from this table that the end titre secured by this method is on the whole higher than

that obtained when using the other methods. The average titre of the complement before splitting for these experiments was 1-180, while the titre after splitting, when the cells were added last was 1-110 and when sensitized cells were used, 1-90. This represents a loss of one-third of the complement activity when cells were added last and of one-half when the cells were sensitized. These results conform more closely to those of Braun. The interesting point to be noted here is that in the case where the best results are obtained, - complete restoration of activity after splitting when non-sensitized cells were used - neither the midpiece nor the endpiece was hemolytic in itself. In fact, in one case only was the hemolysis of either piece at all noticeable. These results are not at all in accord with the observations and conclusions of Kiss. He maintains that it is impossible to split complement and that the so-called splitting is only a dividing of the whole complement into unequal parts. He claims that if it were not for technical difficulties the whole complement would come down with the precipitate. But since this end is never accomplished, there is complement remaining in the endpiece and therefore this is hemolytic in itself, often to a very high degree. The midpiece is usually hemolytic in itself also, particularly when the serum

is diluted 1-20 before adding the CO_2 water. From his experiments he concluded that in order to obtain satisfactory hemolysis after the so-called splitting it is necessary that both midpiece and endpiece be hemolytic in themselves to a high degree. Otherwise the loss in complementing activity is great.

In making our experiments great care was taken to use distilled water through which CO_2 had been passed for some time in order to be sure that the saturation was as complete as possible. This may have been the reason why the pieces were not very hemolytic in themselves. But even under these conditions the precipitation of the globulins was not as complete as when the other methods were used. This was shown by the fact that upon standing a heavy precipitate formed in the endpieces when the Kiss method was used, while in the endpieces obtained from the other methods only a slight precipitate formed, if any at all.

Quantitative Relation of Midpiece and Endpiece: A number of experiments were carried out in an attempt to determine the quantitative relationship existing between midpiece and endpiece. In general, the maximum hemolysis took place when equal amounts of both pieces were used, but such a condition was not entirely essential. It was found that the endpiece was more necessary to hemolysis

than the midpiece and the latter could be decreased in amount very decidedly if there were sufficient of the former present. If relatively large amounts of the midpiece were present the endpiece might also be decreased, but never to the extent that the midpiece might. These results are in accord with those of Braun, Bessemans (1), Marks, and Fränkel (12). Ledingham and Dean, however, while they were able to demonstrate that if sufficient midpiece were present it was possible to decrease the endpiece to some extent, also observed that excessive amounts of midpiece inhibited hemolysis. This was also indicated in our results.

Effect of Midpiece and Endpiece on Normal Complement: The effect of the fractions on normal complement was determined. The endpiece under no circumstances really inhibited the action of normal complement, although it retarded the reaction somewhat at times. The midpiece from freshly fractioned serum retarded the action of normal complement for some time, but did not prevent hemolysis from taking place within thirty minutes. However, when modification of the midpiece has taken place it becomes very inhibitory to normal complement, preventing hemolysis in as small amounts as 1/25 cc. If the serum is heated at 55° C. for 30 minutes before fractioning, the

midpiece does not acquire this anticomplementary property upon standing in isotonic sodium chloride.

Thermostability of Midpiece and Endpiece: Ferrata split guinea pig serum by means of dialysis and studied the stability of the two pieces toward heat. He found that the endpiece was destroyed by heating at 55°C. for 30 minutes, while the midpiece was stable at that temperature. Brand, in repeating the work of Ferrata, found that both midpiece and endpiece were destroyed by heating at 55°C. for 30 minutes. These latter results were obtained by Fränkel and Tsurusaki (35). Marks (25), in 1911, did very extensive work on this point. He found that both pieces when heated after splitting were labile at 55°C. for 30 minutes. He heated normal guinea pig serum at this temperature and found that it could be reactivated by small amounts of normal endpiece but not at all by normal midpiece. In order to determine which was the stable component in this complement, he fractioned the heated serum, using the Sachs HCl method mostly, but also the Liefmann CO_2. The midpiece from the heated serum could be activated by normal endpiece but the endpiece could not be activated by normal midpiece. He therefore concluded that while the midpiece is destroyed by heating at 55°C. for 30 minutes when isolated, it is stable at that temperature when heated under natural con-

ditions. These results were confirmed by Mutermilch (27, 28) and Husler (16).

We attempted to confirm the results of Marks but without success. The endpiece could not be reactivated even by large amounts of serum heated at 55°C. for 30 minutes, although slight hemolysis was obtained with serum which had been heated at 55°C. for only 10 minutes. Although the midpiece from serum that has been heated goes into solution more readily than that from unheated serum, it was never possible to obtain any hemolysis whatever with it and the endpiece from unheated serum, or from any combination of the components of the two types of serum. Persensitized cells were used with entirely negative results.

In 1924 Takenomata (33) reported the results of many experiments performed on this point. He used also both the HCl and the Liefmann CO_2 methods. He found that the midpiece was more resistant when diluted. He also found that at times a lower temperature was more destructive than a higher one. In some cases the midpiece was found to be resistant and in others not. His results on the whole were so inconsistent that he drew the conclusion that the thermoresistance of different midpieces is an extremely variable quantity.

Summary: Guinea pig complement when split into midpiece and endpiece by means of CO_2 loses from two-thirds to one-half of its activity, depending on the method used for splitting. A modification of the Liefmann and Kiss methods was found to give the most satisfactory results under all conditions. The maximum hemolysis is obtained when the fractions are mixed in a 1-1 ratio. The midpiece goes into modification upon standing in isotonic sodium chloride solution and it is impossible to obtain hemolysis with a mixture of midpiece and endpiece. However, hemolysis may be obtained by means of persensitized cells. Both midpiece and endpiece are destroyed by heating at 55° C. for thirty minutes.

Splitting of Guinea Pig Complement into Three Pieces

Method: The method of Browning and Mackie (5) was used in splitting the complement into three pieces. A given volume of guinea pig serum is diluted with three volumes of 0.85% sodium chloride and made up to 33% saturation with a saturated solution of $(NH_4)_2SO_4$ which is added slowly with constant stirring. The euglobulin portion, which is precipitated out of solution at this concentration, is not very large in amount and is centrifuged off immediately and the clear, slightly colored supernatant liquid left for further saturation. The euglobulin

precipitate is dissolved in distilled water equal in amount to the diluted serum and reprecipitated by making up to 33% saturation, and centrifuged. The precipitate is then dissolved in a small amount of distilled water and dialysed over night against running water. The liquid remaining after the precipitation of the euglobulins is made up to 50% saturation by adding the $(NH_4)_2SO_4$ solution in an amount equal to that added to make the 33% saturation. The heavy precipitate of pseudoglobulins formed is centrifuged off immediately. Since not all of the precipitate will come down in the centrifuge, it is necessary to filter it off through a hardened filter paper and add it to the pseudoglobulin fraction when it is dissolved before being reprecipitated and treated as the euglobulin fraction -viz. dialysed over night. Some of this pseudoglobulin does not come down in the centrifuge after reprecipitation, but this is usually discarded. The liquid remaining after these two precipitations is completely saturated with $(NH_4)2SO_4$ crystals and the resulting heavy precipitate of albumins filtered off by suction through hardened filter paper. This is dissolved in a small amount of isotonic sodium chloride and dialysed over night. The color of the serum remains with the albumin fraction. Upon

dialysation the euglobulin and the pseudoglobulin[1] fractions precipitate out of solution while the albumin remains in solution. Each piece is made isotonic with sodium chloride crystals before use and the two globulin fractions are used in dilutions of 1 in 5, while the albumin is used in a 1 in 10 dilution. When separation is complete the pseudoglobulin fraction goes completely into solution when made isotonic, but the euglobulin solution is always cloudy. As $(NH_4)_2SO_4$ is very anticomplementary it is necessary that it be entirely removed during dialysis, and to aid this all precipitates should be made as dry as possible after centrifuging as well as after filtration.

Conclusions of Browning and Mackie: After splitting complement by this method Browning and Mackie claim that they obtained a pseudoglobulin fraction which was hemolytic in itself, frequently to the extent of native serum, but usually the addition of the albumin frac-

1. According to the chemical definition, the euglobulins of the serum are insoluble in water and are precipitated upon dialysis, while the pseudoglobulins are soluble and therefore remain in solution. However, the proteins change their solubilities with great ease (Mathews Physiological Chemistry, third edition, p. 552-553). In the experiments here carried out a precipitation upon dialysis of both the so-called euglobulin and pseudoglobulin fractions obtained by salting out with a saturated solution of $(NH_4)_2SO_4$ is essential for satisfactory results.

tion is necessary in order to obtain the full complementing activity. The addition of the euglobulin fraction did not seem to increase the extent of the reaction. Neither the euglobulin nor the albumin was active in itself, although together they possessed a slight complementing activity.

Experimental: The following table shows the results of some of our experiments.

Table III

This table shows the titre of complement before splitting with $(NH_4)_2SO_4$ and also after splitting when the three pieces and only the pseudoglobulin and albumin are mixed. The sign -- indicates that no tests were made.

No.	Titre of Complement before Splitting	Titre of Conplement after Splitting			
		3 Pieces Mixed		Pseudogl.& Albumin	
		Cells Added Last	Sensitized Cells	Cells Added Last	Sensitized Cells
1	1-400	1-14	1-15	--	--
2	--	1-40	--	1-70	--
3	1-70	1-35	1-20	1-20	1-20
4	--	1-20	1-12	1-20	1-18
5	--	1-28	1-25	--	--
6	--	1-35	--	1-50	--
7	--	1-40	--	1-35	--
8	--	1-18	--	1-12	--
9	--	1-38	--	1-38	--
10	1-200	1-33	1-28	--	--
11	1-250	1-50	1-45	--	--
12	1-200	1-25	1-25	--	--

From the above table it is seen that we were not able to confirm quantitatively the results of Browning and

Mackie. The loss of complementing activity even in the most satisfactory experiments was very great. In order to obtain results even as satisfactory as these it was necessary that the method outlined above be carried out in the shortest possible time. It can usually be completed - up to the point of dialysis- within two and one-half hours. If the various precipitates stand long, some change seems to take place and hemolysis will not be obtained. But there are times when even with the greatest care hemolysis will not take place with the mixing of the three fractions. The reason for this is not clear, but it seems to be in some way connected with the manner in which the euglobulin precipitate comes down. There are times, possibly connected with too vigorous stirring, when only a small portion of the euglobulin precipitates out, and if this is the case the pseudoglobulin fraction will not go into clear solution when the salt solution is added, and it is certain that no hemolysis will take place when the pieces are mixed. It seems as if the euglobulin and pseudoglobulin fractions had not been completely separated, and that the euglobulin may have an inhibiting action on the pseudoglobulin, which latter is evidently the most important of the three fractions. The theory that the euglobulin becomes inhibitory at times is strengthened by the fact that when no hemoly-

sis is obtained by a mixture of the three pieces the euglobulin is apt to inhibit greatly the action of normal complement. This inhibitory action of the euglobulin portion of serum was first noted by Friedemann (13). When separation is incomplete the pseudoglobulin fraction, although it does not inhibit the action of normal complement completely, may yet delay it considerably. When good hemolysis is obtained by mixing the fractions, the euglobulin does not inhibit the action of normal complement, or only slightly, and the pseudoglobulin does not inhibit, but may accelerate. Under no condition does the albumin inhibit the reaction. On the contrary, it frequently accelerates it.

We found, as did Browning and Mackie, that the pseudoglobulin fraction is the only one of the three that is ever hemolytic in itself, and there seems to be no regularity in this property making its appearance. The extent to which this fraction is hemolytic varies greatly, the greatest ever found by us being in a 1-12 dilution. Although hemolysis resulting on the mixing of the three fractions does not seem to be dependent on this hemolytic property of the pseudoglobulin, yet hemolysis will take place to a greater extent if the pseudoglobulin is hemolytic than if it is not. Neither the euglobulin nor the albumin fractions are ever hemolytic in themselves or

when mixed together. Whether or not the euglobulin and the pseudoglobulin are hemolytic when mixed will depend on the ability of the pseudoglobulin to cause hemolysis in itself. The addition of the euglobulin increases the activity of the pseudoglobulin, - for example, from a 1-12 to a 1-25 dilution. It will be seen from table III that when hemolysis takes place after the mixing of the three fractions it will also take place when the pseudoglobulin and the albumin alone are mixed. A comparison of the hemolysis obtained under these two conditions, as seen in table III, will show that the addition of the euglobulin does not as a rule increase the extent of hemolysis obtainable. Occasionally the hemolysis is greater with the two fractions showing the inhibitory effect of the euglobulin. In all experiments it made very little difference whether the cells were added last or first sensitized.

The following protocol, II a and b, are those used in setting up these experiments (see page 26).

The length of time during which the fractions remain active after preparation is an extremely variable quantity. It may be said in general of both the three pieces and the two pieces that their ability to cause hemolysis when mixed decreases steadily. In some

Protocol II

Type used in determining the hemolytic activity of complement after splitting with $(NH_4)_2SO_4$.

a	Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13
	Sensitizer 5 Units	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Euglobulin(1-5) Pseudoglobulin(1-5) Albumin(1-10)	1.6	1.4	1.2	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
	Salt Solution	0.2	0.4	0.6	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7
						Incubate 20 minutes at 37° C.								
	Sheep Cells (1-4)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
						Incubate 60 minutes at 37° C.								

b	Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13
	Sensitizer 5 Units	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Sheep Cells(1-4)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Salt Solution	0.2	0.4	0.6	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7
						Incubate 20 minutes at 37° C.								
	Euglobulin(1-5) Pseudoglobulin(1-5) Albumin(1-10)	1.6	1.4	1.2	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
						Incubate 60 minutes at 37° C.								

cases it has disappeared in 3-4 hours, while in others it is still present to a slight extent after 24 hours. The pieces deteriorate more rapidly if they stand separately and are mixed just before using, than if they stand mixed for some hours. It would seem that the three pieces would lose their ability to cause hemolysis when mixed more quickly than the two, because of the presence of the euglobulin, but this was not completely demonstrated. The small amount of data collected shows that all euglobulin fractions do not become more anticomplementary as they stand.

The Water as a Disturbing Factor in Dialysis: In the late fall of 1924 after having consistently obtained good results for about a month, it was noticed that the hemolysis obtained after fractioning became less and less until it was impossible to obtain any results whatsoever. As this condition continued to exist new materials were obtained and used with negative results. It was finally found that during the process of dialysis the euglobulin and pseudoglobulin failed to precipitate out of solution. It appeared as if some of the proteins had been changed to such an extent that they had passed through the membrane, for upon precipitation with $(NH_4)_2SO_4$ it did not seem that the normal amount of precipitate was formed. If dialysis were made against

distilled water instead of against tap water both the euglobulin and the pseudoglobulin were completely precipitated out of solution.

The Baltimore water supply is filtered and treated with chemicals before it is used. During the fall of 1924 there was a fungus growth in the water and in order to kill this manganese was added and then aluminium sulphate to precipitate the manganese. This excess of sulphate may have been sufficient to prevent the proteins from precipitating out of solution. However, manganese is only added to the water for two months in the fall and the inability to obtain a precipitate upon dialysis has persisted for five months with an interruption of only about two weeks. Examination of the composite analyses of the water made by the city each month shows a very high bicarbonate content of the water during the months of November and December when conditions were at their worst. When a comparison is made between the analyses for these two months and those for April and May, when very good results were obtained, this excess of bicarbonates and a slight excess of calcium seem to explain our inability to precipitate the proteins upon dialysis.

An attempt was made to carry out the dialysis against distilled water instead of against tap water, but

no satisfactory results were obtained. As mentioned above, the precipitation of the proteins was excellent, but no matter how many times the water was changed the euglobulin or the pseudoglobulin, or both, were extremely anticomplementary in themselves and therefore it was impossible to obtain hemolysis when they were mixed. From observations made during the course of some other experiments it appears that this is due to some anti-complementary action of the distilled water itself on the fractions and not to incomplete removal of the $(NH_4)_2SO_4$.

Since this difficulty with dialysis has been encountered, our inability to obtain results equivalent to those of Browning and Mackie perhaps may be due to differences in the water supplies used in the two cases.

Summary: Guinea pig complement may be split into euglobulin, pseudoblobulin, and albumin fractions by means of a saturated solution of $(NH_4)_2SO_4$. The loss of complementing activity in our experiments was very great, contrary to the claims of Browning and Mackie. This may have been in part due to the difference in the water supplies used for dialysis.

Splitting of Guinea Pig Complement into Four Pieces

Method: In an attempt to fraction guinea pig serum into four pieces the method used was that employed by Browning and Mackie. That is, the serum was first split into midpiece and endpiece by the Liefmann CO_2 method, then by use of $(NH_4)_2SO_4$ the midpiece was split into euglobulin and pseudoglobulin and the endpiece into pseudoglobulin and albumin.

The CO_2 fractioning was carried out as described under fractioning of complement into two pieces, except that the endpiece was not made isotonic after the removal of the midpiece. The mid piece was dissolved completely before the addition of the $(NH_4)_2SO_4$. The splitting of the midpiece was the most difficult and probably the most important part of the fractioning. The euglobulin was precipitated by 33% saturation, and the pseudoglobulin by 50% saturation as described in detail under three piece fractioning. But the separation of these two precipitates seems to be very difficult to accomplish successfully. The amount of both is small, that of the latter very small, and the addition of the $(NH_4)_2SO_4$ seems to cause them to precipitate differently at different times. As a rule, the euglobulin frac-

tion will come down in the centrifuge, but the pseudoglobulin never comes down completely, even after reprecipitation, and must be filtered off both times through hardened filter paper. Upon dialysation both precipitate out somewhat, and when sodium chloride is added to isotonicity neither solution becomes entirely clear.

The fractionation of the endpiece is accomplished quite readily and the pseudoglobulin and albumin precipitates, obtained by 50% and 100% saturation with $(NH_4)_2SO_4$, behave exactly as under the three piece fractioning and are treated in the same way. The amount of pseudoglobulin fraction is somewhat smaller than in the three piece fractioning, due to the small portion of it that goes with the midpiece.

Conclusions of Browning and Mackie: Browning and Mackie summarize their results from the four piece fractioning as follows: The pseudoglobulin from the midpiece is the smallest part of the serum, but is the only one which has any complementing activity in itself. The two pseudoglobulin fractions together show a marked hemolytic activity, which at times may be equal to that of native serum. However, the addition of the albumin of the serum is usually necessary to restore complete activity. The addition of the euglobulin did not increase the activity of the pseudoglobulins. The albumin as a rule

is incapable of acting with the pseudoglobulin of the endpiece, but has a marked action together with the pseudoglobulin of the midpiece. It is also somewhat active with the euglobulin, although the addition of a third component is usually necessary for the restoration of complete activity.

Discussion: The results obtained by us insecuring hemolysis when mixing the four fractions together have been entirely negative. Only once has any hemolysis been obtained and then only to a small degree with the mixture of the two pseudoglobulin fractions and the albumin. In making up the protocols both sensitized and unsensitized cells were used and as much as 1.6 cc. of the mixture.

In trying to determine the reason for these negative results the effect of the various fractions on normal complement was tried. In only one out of eight cases did the euglobulin inhibit the action of normal complement. In all eight cases the pseudoglobulin from the midpiece was inhibitory, in some instances more so than in others. In no case did either the pseudoglobulin from the endpiece or the albumin fractions cause any inhibition, but in several cases they actually caused an acceleration. It might therefore be supposed that this inhibition which has developed in the pseudoglobulin

from the midpiece may in some way prevent hemolysis from taking place when the fractions are mixed in various ways.

In view of the great difficulty which has been experienced with changes in the city water supply it seems possible that herein might lie in part the reason for our inability to obtain the results of Browning and Mackie.

Reactivation of Yeast Absorbed Guinea Pig Complement

Three Piece Fractions.

Experimental: Fresh guinea pig serum was mixed with a small amount of Fleischmann's yeast and kept at 37°C. for 30 minutes. The yeast was then removed by centrifuging. The serum thus obtained was diluted 1-5 with isotonic sodium chloride solution and was found to be completely inactive when 0.1 cc. of this dilution was added to two units of sensitizer and one unit of sheep cells. It is best to use fresh yeast since, unless it is dried, it may become anticomplementary on aging.

In reactivating this yeast-absorbed serum with the fractions from the three piece $(NH_4)_2SO_4$ fractioning the results were very clear cut. Neither the euglobulin nor the albumin fraction activated the serum in the least, while a complete and rapid activation took place with very

small amounts of the pseudoglobulin fraction. Protocol III, a and b, were those used in testing for the reactivation. In many cases it was necessary to carry the pseudoglobulin fraction into higher dilutions in order to determine the greatest extent to which reactivation would take place.

The reactivating power of the pseudoglobulin fraction was determined before and after heating. In a series of experiments, as shown in table IV, only the results with the pseudoglobulin fraction are included as no reactivation was ever obtained with the euglobulin or the albumin fractions.

Table IV

This table shows the results obtained in reactivating the yeast absorbed complement with the pseudoglobulin fraction from $(NH_4)_2SO_4$ splitting. -- = no test. 0 = no reactivation.

No.	Unheated Pseudoglobulin		Heated Pseudoglobulin	
	Cells Added Last	Sensitized Cells	Cells Added Last	Sensitized Cells
1	1-50	1-25	--	--
			56°C. 30'	
2	1-50	1-50	1-25	1-50
3	1-50	1-25	1- 9	1- 5 ±
4	1-50	1-30	--	--
5	1-50	1-40	0	0
6	1-50	1-25	--	--
7	1-125	1-50	--	--
			54°C. 30'	
8	1-95	1-65	1-25	1- 3±
9	1-85	1-50	1-25	1-12
10	1-50	1-25	0	0
11	1-50	1-40	1-25	1-13
12	1-65	1-60	1-30	1-25

Protocol III

Type used in reactivating yeast absorbed complement with fractions from splitting with $(NH_4)_2SO_4$.

Euglobulin 1-5
Pseudoglobulin 1-5
Albumin 1-10

a	Tubes	1	2	3	4	5	6	7	8	9	10
	Sensitizer 5 Units	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Yeast Absorbed Complement (1-5)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Fraction	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
	Salt Solution	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6
					Incubate for 20 minutes at 37° C.						
	Sheep Cells (1-4)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
					Incubate for 60 minutes at 37° C.						

b	Tubes	1	2	3	4	5	6	7	8	9	10
	Sensitizer 5 Units	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Sheep Cells(1-4)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Yeast Absorbed Complement(1-5)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Salt Solution	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6
					Incubate for 20 minutes at 37° C.						
	Fraction	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
					Incubate for 60 minutes at 37° C.						

Analysis of Table IV: As seen in the above table, the extent to which the various pseudoglobulin fractions will reactivate yeast absorbed guinea pig serum varies greatly. But it is definitely seen that reactivation takes place to a greater extent when cells are added last than when sensitized cells are used. It was thought that possibly the extent to which the pseudoglobulin fraction would reactivate the yeast absorbed serum was dependent on the property which this fraction sometimes possessed of being hemolytic in itself. This was found, however, not to be the case. Only in four cases was the pseudoglobulin hemolytic in itself and then never in more than a 1-8 dilution. The reactivation possible with these four fractions was in no case greater than with the nonhemolytic fractions, and usually not as great.

After heating the pseudoglobulin fraction, either at 56°C. or at 54°C. for 30 minutes, a decided decrease in its ability to reactivate the yeast absorbed serum takes place, but it is not as a rule entirely destroyed. The same relationship between the two orders of addition obtains as when unheated pseudoglobulin is used. It has been shown by Hyde (18) that it is possible to reactivate yeast absorbed serum with small amounts of guinea pig complement heated at 56°C. for 30 minutes. Since

the pseudoglobulin fraction loses in part its ability to reactivate the yeast absorbed serum when heated at that temperature, it is probable that this is due to its isolated condition.

Two Piece Fractions.

Experimental: In the reactivation of the yeast absorbed complement with fractions from the three piece $(NH_4)_2SO_4$ fractionation is was found that the pseudoglobulin was the one which contained the substance necessary for reactivation. In the light of this fact one would suppose that since both the endpiece and the midpiece obtained from fractioning guinea pig complement with CO_2 contain pseudoglobulin that both of these pieces would possess the property of reactivating the yeast absorbed serum. This supposition has been found to be true as is seen from the following table V, which gives the results of twelve experiments. The same dilutions of the substances and the same protocols were used as in reactivating with the three piece fractions. It may be noted here that the results were not as clear cut as with the three pieces, and many of the reactions were extremely difficult to read. This was particularly true after heating. In cases where there is doubt (±) is inserted after the reading.

Table V

This table shows the reactivation of yeast absorbed complement with midpiece and endpiece from CO_2 fractioning. 0 = no reactivation. -- = no test made.

No.	Midpiece				Endpiece			
	Unheated		54°C.30'		Unheated		54°C.30'	
	Cells Added Last	Sensitized Cells	Cells Added Last	Sensitized Cells	Cells Added Last	Sensitized Cells	Cells Added Last	Sensitized Cells
1	1- 50	1-50	0	1- 3	1- 3	0	0	0
2	0	1-50(±)	0	1-25(★)	1-10	0	0	0
3	0	1- 3	0	0	1- 9	1- 4	0	0
4	1- 50	1-30	0	0	1-15	1-10(±)	1- 3	0
5	1- 50	1-25	0	1-10(±)	1-12	1-2	0	0
6	1- 50	1-50	--	--	1-17	1-5(±)	--	--
7	1- 50	1-40(±)	1-40	1-17(±)	1-17	1-4	1-10	--
8	1- 30	1-17	0	1- 4	1- 6	0	0	0
9	1- 85	1-12	1-60	1-12(±)	1-12	0	1- 8	0
10	1-125	1-125	1-80	1-80(±)	1-20	1-12	1-12	1-5
11	1- 40	0	1-30(±)	1-25(±)	1-12	1- 4	1- 6	0
12	1- 70	1- 35	1-50	1-30	1- 5	0	0	0

Analysis of Table V: The splitting of the pseudo-globulins present in the whole serum by means of CO_2 very definitely affects their ability to reactivate the yeast absorbed serum for such reactivation is not at all clear cut. For the most part the reactivating property goes with the midpiece and the end point of the reaction may be as high as in the case of

the unsplit pseudoglobulins. Yet the endpiece also possesses some ability to reactivate. It must be remembered in this connection that the loss of complementing activity in fractioning guinea pig serum into three pieces is greater than when fractioning it into two. This appears to account for the fact that, as shown by comparing tables IV and V, the split pseudoglobulin may reactivate to as great an extent as the unsplit. The midpiece, however, does not reactivate as consistently in the higher dilutions as does the unsplit pseudoglobulin, and as mentioned before the reactions are not as clear cut.

The two orders of addition show that the reactivation takes place better when cells are added last than when sensitized cells are used. This was also found true in reactivating with the pseudoglobulin. Heating of the midpiece and endpiece at 54°C. for 30 minutes either reduces the extent to which the reactivation will take place or destroys it entirely. There seems to be no way of determining why the reactivating ability is destroyed in some cases and not in others. Experience with fractioning the guinea pig serum into three pieces has shown that the euglobulin fraction of the serum is very apt to become anticomplementary during the process. It has been observed that it is almost always in reactivating with the midpiece that the results are not clear cut and are difficult

to read. This lack of clearness in the reactions is particularly noticeable after the midpiece is heated. It is possible that during the process of fractioning and heating, the euglobulin sometimes becomes anticomplementary and thus inhibits to some extent the action of the pseudoglobulin present in the midpiece.

Discussion and Summary: Von Dungern (8) was the first to show that serum could be inactivated by contact with yeast, and it was later used by Ehrlich and Sachs (9, 10) in their demonstration of "Complementoid". These latter showed that the action of yeast was in removing a component of complement and this was confirmed by the work of Coca (6). He found that serum treated with yeast was activated with both the midpiece and endpiece of complement obtained from splitting with CO_2 and therefore the component removed was independent of these pieces as such; that is, yeast absorbed complement contained both midpiece and endpiece. It was also resistant to heat. Hyde (18) demonstrated that yeast absorbed serum could be activated with heated guinea pig serum and human serum, the order of the addition of the reagents used being extremely important in obtaining satisfactory results.

Much work has been done on the inactivation of guinea pig serum with cobra venom. Sachs and Omorokow(31)

found that serum treated in this way could be activated with the endpiece and midpiece of complement. Ritz (29) found this to be true and in addition demonstrated that it was possible to activate serum heated at 54°C. for 30 minutes by means of serum inactivated with cobra venom. Midpiece and endpiece obtained by splitting serum with CO_2 when heated at 54°C. for 30 minutes would also activate the serum treated with cobra venom. Husler (16) confirmed these results. From the above facts these workers considered the presence of a third component in serum demonstrated. Coca states that a comparison of the properties possessed by sera treated with yeast and cobra venom definitely shows the identity of the component removed by the two substances; that is, the third complement component. In the case of cobra venom, however, it is not a removal but rather an inhibition of the function, for it is possible, though only at times, to reactivate the yeast absorbed serum with small amounts of the cobra venom inactivated serum.

Ritz was able to demonstrate in the case of serum inactivated with heat and then fractioned that the stable third component adhered to the globulin portion of the serum. Takenomata (33) in his work has been able to confirm this with active serum. Our results in connection with the reactivation of the yeast absorbed serum have

shown that this is the case. But we have gone further and in splitting the guinea pig serum into three pieces as well as two, have been able to show that the third component is all in the pseudoglobulin fraction and entirely missing in the euglobulin fraction. The relative resistance of this pseudoglobulin fraction to heat shows more definitely that it is identified with the third complement component. The yeast evidently removes a component found in the pseudoglobulin fraction. Since the deficient guinea pig serum will not reactivate the yeast absorbed serum they would seem to have at least one complement component missing in common.

Activation of Deficient Guinea Pig Serum Two Piece Fractions.

Experimental: There is a colony of guinea pigs in the possession of R. R. Hyde which are markedly deficient in the serum constituent called complement. This complement deficiency is an inherited character and behaves as a simple recessive Mendelian unit. The inheritance is the same whether transmitted through the male or female. In order to determine what piece has been removed from this complement by nature, attempts were made to activate it by means of the pieces obtained from the two and three piece fractionings.

Protocol IV, a and b, were those used in the activating of the deficient guinea pig serum with midpiece and endpiece obtained from fractioning guinea pig complement with carbon dioxide. Five units of sensitizer were used and the deficient serum diluted 1 in 5 with isotonic sodium chloride. It was usually necessary to make further dilutions of the endpiece than are shown in the protocols in order to obtain the endpoint of the reaction. These protocols illustrate the two methods of addition used.

The following table, VI, summarizes the results obtained from several experiments. The results of the activation of the deficient serum before and after heating at 54°C. for 30 minutes are given. The serum from seven different deficient pigs was used in these experiments.

Analysis of Table VI: The principal fact in this table is that the substance most important in the activation of deficient guinea pig serum is present in the endpiece of complement, obtained by fractioning with CO_2, to the greatest extent. The extent to which the endpiece will activate the deficient serum varies greatly with the different fractionings. Activation was obtained anywhere from a 1-35 to a 1-5000 dilution. It will

Protocol IV

Type used in showing the activation of deficient guinea pig serum with midpiece and endpiece from CO_2 fractioning.

a	Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Sensitizer 5 Units	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Deficient Serum (1-5)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Midpiece or Endpiece	(1-5)* 1.0	0.8	0.6	0.5	0.4	0.3	0.2	(1-25) 0.8	0.6	0.5	0.4	0.3	0.2	0.1
	Salt Solution	0.7	0.9	1.1	1.2	1.3	1.4	1.5	0.9	1.1	1.2	1.3	1.4	1.5	1.6
						Incubate 20 minutes at 37°C.									
	Sheep Cells 1-4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
						Incubate 60 minutes at 37°C.									

b	Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Sensitizer 5 Units	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Sheep Cells (1-4)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Deficient Serum (1-5)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Salt Solution	0.7	0.9	1.1	1.2	1.3	1.4	1.5	0.9	1.1	1.2	1.3	1.4	1.5	1.6
						Incubate 20 minutes at 37° C.									
	Midpiece or Endpiece	(1-5)* 1.0	0.8	0.6	0.5	0.4	0.3	0.2	(1-25) 0.8	0.6	0.5	0.4	0.3	0.2	0.1
						Incubate 60 minutes at 37° C.									

*(The figures in brackets indicate the dilution of the fraction before setting up the protocol.)

Table VI

This table shows the results obtained in activating deficient guinea pig complement with midpiece and endpiece from CO_2 fractioning. ± = doubtful readings. -- = no test made. 0 = no activation.

No.	Midpiece				Endpiece			
	Cells Added Last		Sensitized Cells		Cells Added last		Sensitized Cells	
	Unheated	54°30'	Unheated	54°30'	Unheated	54°30'	Unheated	54°30'
1	1-50	0	1-30	0	1-35	0	0	0
2	0	0	0	0	1-1000	0	1-3	0
3	1-85	--	1-50	--	1-5000	0	0	0
4	1-75	0	1-30	0	1-1000	0	0	0
5	0	0	0	0	1-2500	0	1-8	0
6	0	0	0	0	1-85	0	1-7	0
7	1-50	0	1-30	0	1-420	0	0	0
8	0	0	0	0	1-85	0	0	0
9	1-25(±)	0	1-12(±)	0	1-45	0	0	0
10	0	0	0	0	1-2500	0	1-50(±)	0
11	0	0	0	0	1-3000	0	1-800	0
12	1-75	0	1-40	0	1-3000	0	1-800	0

also be noticed that the method of addition of the substances used is very important in this case. If the cells are added last activation takes place, while if sensitized cells are used no activation takes place, or to such a slight extent, as a rule, that it may be neglected. In two cases activation was obtained in a 1-800 dilution when using sensitized cells. In these cases a sensitizer which was not more than two weeks old was used, while in the other cases older serum of high titre was used. It was thought possible that this might have some effect on the results obtained, but further experiments with perfectly fresh sensitizer seem to show that this is not the case. It is impossible to explain these facts from the observations we have made. However, in the cases in which the midpiece activates, results are obtained with both orders of addition under all conditions. This irregularity in securing activation with sensitized cells is particularly important in view of the fact that sensitized cells were used entirely by all the earlier workers in all of their experiments. If sensitized cells alone had been used in the above experiments activation would not have been secured in the majority of the cases.

The endpiece retains its ability to activate deficient guinea pig serum for some time. One endpiece

which when fresh activated in a 1-420 dilution, after standing in the ice box for ten days activated in a 1-300 dilution and did not lose its ability completely for nearly a month. Others have retained their ability even longer. It is interesting to note that in the cases in which distilled water saturated with CO_2 was used the endpiece obtained loses its ability to activate deficient serum more quickly. A very heavy precipitate forms in this case. However, if the other method of fractioning is used, but a slight one forms. A few of the endpieces upon standing acquired the ability to activate when sensitized cells were used, but never to as great an extent as when cells were added last.

It will be noted on examining table VI that in half of the cases the deficient serum is activated by the midpiece as well as the endpiece, but never to as great an extent -- in a 1-85 dilution being the greatest ever obtained. This activation of the serum by the midpiece does not seem to bear any relationship to the extent of activation produced by the endpiece. It appears that it takes place when the serum is so split that more of the pseudoglobulin is in the midpiece than normally and is more or less of a chance affair. When the midpiece activates the deficient serum it does so in both orders of

addition, although as great activation is not obtained when sensitized cells are used as when the cells are added last. Whatever the substance which activates the deficient serum in either mid piece or endpiece, it loses its ability entirely when heated at 54°C. for 30 minutes.

Discussion: Coca (7) and Hyde (17,19) have attributed the inactivity of the deficient guinea pig serum to a lack of a third complement component. Their contention is that both midpiece and endpiece are present in the deficient serum. The third component is supplied by normal guinea pig serum, resists heating at 56°C., and is absorbable by yeast. The fact that both midpiece and endpiece lost their power to activate the deficient serum when heated at 54° C. for 30 minutes indicates that the third component is more easily affected by temperature when removed from its native condition. The fact that the yeast removes a complement component in the pseudoglobulin fraction and that the deficient guinea pig serum is not activated with yeast absorbed complement indicates very definitely that at least one of the factors missing in the deficient guinea pig complement is associated with the pseudoglobulin fraction.

Three Piece Fractions.

Experimental: The work of activating deficient guinea pig complement by means of three fractions from the

$(NH_4)_2SO_4$ fractioning has not been at all satisfactory or conclusive. The reason for this is that shortly after this work was begun the changes in the water supply, described above, began to take place and it was impossible to obtain satisfactory fractions with which to continue. The observations made from the few experiments carried out are, however, given below.

In the first attempts to activate deficient guinea pig serum it was found that activation would take place by the addition of the albumin fraction and not by the addition of either the euglobulin or the pseudoglobulin. Activation was obtained with the albumin fraction in as high as a 1-500 dilution, and upon heating at 56°C. for 30 minutes activation was obtained only in a 1-300 dilution. These experiments were repeated some months later and it was found that activation either did not take place at all, or upon the addition of both the pseudoglobulin and the albumin fractions, or sometimes with one and not the other. It was not obtained with either in more than a 1-125 dilution.

Discussion: In light of subsequent work in activating the deficient guinea pig serum with two piece fractions it is possible at least to formulate a theory as to the reason for these contradictory results. It was found

in this latter work that although at times both midpiece and endpiece would activate the deficient serum, activation with the former was not constant and never took place to a very high degree, while with the endpiece activation always took place and in very high dilutions. As the endpiece from the CO_2 splitting contains both pseudoglobulin and albumin, a mixture of these two is necessary in order to obtain the most satisfactory results. But since activation is sometimes obtained with the midpiece and this contains no albumin, it is seen that the pseudoglobulin alone possesses the property of activation, although not to a very great extent. No activation of any kind was ever obtained with a euglobulin fraction.

It is possible that the albumin fractions first used by us to activate the deficient guinea pig serum contained some pseudoglobulin and this is the reason activation was obtained to such a high degree. These fractions were made during the early part of our work on splitting complement with $(NH_4)_2SO_4$ and it is highly probable that a complete separation of the two proteins had not taken place. The latter experiments were performed with fractions which were more perfectly separated, and indicate that it is possible to activate the deficient serum with both pseudoglobulin and albumin alone, but a combination of the two is more satisfactory. If the conditions for

dialysis become more satisfactory it is desirable to determine the quantitative relationships between the pseudoglobulin and albumin necessary to obtain the most satisfactory activation. Since the power to activate is entirely destroyed by heating at 54°C. for 30 minutes when both midpiece and endpiece are used, it is desirable, to determine whether both of these fractions, pseudoglobulin and albumin, are thermolabile or whether this property applies to only one.

If one will compare the results obtained in activating yeast absorbed complement and deficient guinea pig complement by means of the two piece and three piece fractions, it will be seen that while the substance which activates the yeast absorbed serum is contained mainly in the midpiece, that which activates the deficient serum is contained mainly in the endpiece. Yeast absorbed serum is activated only by the pseudoglobulin from the three piece fractioning, while the deficient serum is from all indications activated by both the pseudoglobulin and the albumin, and a combination of the two will bring about the most satisfactory activation. These results show that the inactivation of guinea pig complement brought about by absorption with yeast and that produced by nature are not identical.

DISCUSSION

Brand, in his attempt to explain why the midpiece of complement went into modification when standing in salt solution, came to the conclusion that since the salt conditions in the blood were the same as those in the experiment the modification must take place by virtue of the isolation of the midpiece, and that in the blood both components are united as an active complement. From the fact that he was able to demonstrate, by the fractioning of the sera of many animals, that the midpiece of complement is always present while it is the endpiece which is the variable quantity, Braun concluded that the components are not combined in the native serum, but remain separate. Gengou (14) also drew the conclusion from his experiments that these two components are separate in native serum.

Although opinions have differed as to whether or not the components of complement are united or separated in native serum, the great majority of workers have not questioned the possibility of splitting complement into its components. However, there are those who maintain that it is impossible to split complement. Prominent among these are Bronfenbrenner and Noguchi (4). They carried out their experiments using the HCl, the CO_2 and the dialysis methods. They concluded that complement split-

ting by these methods is really inactivation of the whole complement by certain acids or alkalis, either added in the free state to the serum or liberated as the result of the dissociation of certain electrolytes. The whole complement and not a part, they said, is present in the endpiece and may be demonstrated by the removal of the inhibitory effect of the acid or alkali. The midpiece activates the endpiece by virtue of its amphoteric nature. Activation may also be brought about by the addition of other amphoteric substances, such as egg white and alanin. Liefmann (23) attempted to confirm these results, but was unable to do so. He thought that they were due to incomplete splitting of the complement.

Kiss maintains, as has been mentioned, that it is impossible to split complement. If the conditions are ideal all of the complement comes down with the precipitate when the CO_2 gas is passed through the serum. But such perfection is never attained and therefore complement is found in both the midpiece and the endpiece, although in unequal amounts. He explains the difference in the properties of these two pieces as follows: The endpiece contains the complement which during the process of precipitation is dehydrated and not completely activated by the addition of sodium chloride. The midpiece contains the complement which comes down with the globulin preci-

pitate. But it also contains an inhibiting substance or otherwise its properties would be the same as those of the endpiece. The action which the midpiece has on the endpiece is that of bringing to light the hidden complement - a type of regeneration.

In spite of the opinions of Kiss and Bronfenbrenner and Noguchi, the great mass of evidence seems in favor of the theory that it is possible to split complement. Since it is possible to remove certain substances from the complement at will, making it entirely inactive, and then by the addition of certain substances and not others to make it active again, it appears that complement is not one single substance, but is made up of several components. The fact that in the complement deficient guinea pig we have a condition in which a component has been removed by nature, is greatly in favor of the complex constitution of complement. As to the number of components of which complement is made up, no definite statement can be made. Probably the complementing activity of a serum is due to the interaction of a number of factors intimately associated with the protein structure of the serum. Our data seem to indicate that at least three factors are involved.

SUMMARY

1. Guinea pig complement was split into two pieces by means of CO_2. The hemolysis resulting from a mixture of the two fractions showed a loss of about two-thirds of the complement activity. The results of earlier workers as to the quantitative relationships of the two pieces and the modification of the midpiece when standing in sodium chloride were confirmed. Both midpiece and endpiece were found to be labile at 55°C. for 30 minutes.

2. Guinea pig complement was split into three pieces by means of $(NH_4)_2SO_4$ as described by Browning and Mackie. Our results, although less satisfactory quantitatively, confirmed those of the above workers. In light of the difficulties encountered this difference is probably due to the water supplies used for dialysis.

3. Attempts were made to split complement into four pieces by means of CO_2 and $(NH_4)_2SO_4$ as described by Browning and Mackie, but with no success. It is highly probable that the water supply affected these results.

4. Guinea pig complement absorbed with yeast was activated by both the midpiece and endpiece from CO_2 fractioning, but more consistently and to a greater extent by the former. Heating of the pieces at 55°C. for 30 minutes reduced their ability to reactivate. The re-

activation of the yeast absorbed serum by means of the three pieces from the $(NH_4)_2SO_4$ splitting shows this function to lie entirely in the pseudoglobulin fraction. It is partially destroyed by heating at 56°C. for 30 minutes, due to its isolated condition.

5. Serum from the complement deficient guinea pig was always activated by the endpiece from the CO_2 splitting in high dilutions, and by the midpiece frequently, but in lower dilutions. Heating at 55°C. for 30 minutes destroyed the activating ability entirely. The data available indicate that both the pseudoglobulin and the albumin from the $(NH_4)_2SO_4$ splitting will activate the deficient serum to a small extent, but a mixture of the two is essential for the most satisfactory activation.

6. The component removed from complement by the absorption with yeast is not identical with that removed by nature in the complement deficient guinea pig serum.

The writer wishes to express her sincere appreciation to Dr. Roscoe R. Hyde for the constant guidance and valuable suggestions given during the course of this work.

BIBLIOGRAPHY

1. Bessemans, A.:

1913. De l'importance respective des deux constituants de l'alexine dans le phénomène de l'hemolyse.
Zeitschr. f. Immunitätsforsch. 17 p. 36-46.

2. Brand, Erwin:

1907. Ueber das Verhalten der Komplemente bei der Dialyse.
Berl. klin. Wochenschr. 34 p. 1075-1079.

3. Braun, H:

1911. Beiträge zur Kenntnis des Komplementes.
Biochemische Zeitschrift 31 p. 65-112.

4. Bronfenbrenner, J. and Noguchi, H:

1912. A Biochemical Study of the Phenomena Known as Complement Splitting.
Journal Experimental Medicine 15. p. 598-643.

5. Browning, C. H. and Mackie, T.J.:

1914. Studies on Complement Action: With Special Reference to the Fractioning of Complement by Means of Ammonium Sulphate.
Zeitschr. f. Immunitätsforsch. 21 p.422-446.

6. Coca, Arthur F.:

1914. A Study of the Anticomplementary Action of Yeast, of Certain Bacteria, and of Cobra Venom.
Zeitschr. f. Immunitätsforsch. 21 p. 604-622.

7. Coca, Arthur F.:

1920. A Study of the Serum of Complement Deficient Guinea Pigs.
Proc. Exp. Biol. and Med. 18 p. 71.

8. Von Dungern:

1920. Beiträge zur Immunitatslehre.
Münch. med. Wochenschr. 47 p. 677-680.

9. Ehrlich, P. and Sachs, H.:

1902. Ueber die Vielheit der Komplemente des Serums.
Berl. klin. Wochenschr. p. 297-299, 335-338.

10. Ehrlich, P. and Sachs, H.:

1902. Ueber den Mechanismus der Amboceptorenwirkung.
Berl. klin. Wochenschr. p. 492-496.

11. Ferrata, Adolpho:

1907. Die Unwirksamkeit der komplexen Hämolysine in salzfreien Lösungen und ihre Ursache.
Berl. klin. Wochenschr. 13 p. 366-368.

12. Fränkel, Ernst:

1911. Ergebnisse bei der Spaltung des hämolytischen Komplementes in Mittel- und Endstück.
Zeitschr. f. Immunitätsforsch. 8 p.781-800.

13. Friedemann, Ulrich:

1910. Experimentelle Untersuchungen zur Theorie der Wassermannschen Reaktion.
Zeitschr. f. Hygiene 67 p. 279-348.

14. Gengou, Oct.:

1911. Recherches sur la constitution de l'alexine et son absorption par les précipités spécifiques.
Zeitschr. f. Immunitätsforsch. 9 p. 344-358.

15. Hecker, Rudolf:

1907. Beiträge zur Kenntnis der hämolytischen Komplemente.
Arb. aus dem König. Inst. f. Exper. Therapie Heft 3 p 39-60.

16. Husler, Josef:

1912. Ueber die Inaktivierung hämolytischer Komplemente durch Erwärmen. Zeitschr. f. Immunitätsforsch. 15 p.157-171.

17. Hyde, R.R.:

1923. Complement Deficient Guinea Pig Serum. Journal of Immunology 8 p. 267-286.

18. Hyde, R. R.:

1925. The Activation of Yeast Absorbed Serum with Heated Sera. Amer. Jour. of Hygiene 5 p. 145-148.

19. Hyde, R.R.:

1924. The Activation of Complement Deficient Guinea Pig Serum with Heated Sera. Amer. Jour. of Hygiene 4 p. 62-64.

20. Kiss, Julius:

1921. Alexin und Antialexin. III Abschnitt

21. Ledingham, J.C.G. and Dean, H.R.:

1912. The Action of the Complement Fractions on a Tropin-B. typhosus System with Comparative Hemolytic Experiments. Journal of Hygiene 12 p. 152-194.

22. Liefmann, H.:

1909. Ueber den Mechanismus der Seroreaktion der lues. Münch. med. Wochenschr. 41 p. 2097-2101.

23. Liefmann, H.:

1912. Weichhardt's Jahresbericht. 8 (Cited from Zinsser "Infection and Resistance" 3d. ed. p. 200.)

24. Marks, H.R.:

1911. Ueber gewisse qualitative und quantitative Beziehungen zwischen Endstück/Mittelstück und der Komplemente.
Zeitschr. f. Immunitätsf. 8 p. 508-525.

25. Marks, H.R.:

1911. The Thermostability of the Midpiece of Complement.
Zeitschr. f. Immunitätsf. 11 p. 18-30.

26. Michaelis, Leonor, and Skwirsky, P.:

1910. Der Einfluss der Reaktion auf die spezifische Hämolyse.
Zeitschr. f. Immunitätsf. 4 p. 357-374, 629-635.

27. Mutermilch, Stéfan:

1911. Syr la dissociation de l'alexine dans les sérum inactivés par la chaleur.
Comp. Rendus de la Societé de Biologie 70 p. 577.

28. Mutermilch, Stéfan:

1911. Sur la dissociation de l'alexine dans les viuex sérum inactivés.
Comp. Rendus de la Societé de Biologie 71 p. 605.

29. Ritz, H.:

1912. Ueber die Wirkung des Cobragiftes auf die Komplemente.
Zeitschr. f. Immunitätsf. 13 p. 62-83.

30. Sachs, H. and Altmann:

Cited from Kolle und Wassermann Handbuch 2 p. 710.

31. Sachs, H. and Omorokow, L:

1911. Ueber die Wirkung des Cobragiftes auf die Komplemente. Zeitschr. f. Immunitätsf. 11 p. 710-724.

32. Sachs and Teruuchi, Y.:

1907. Die Inactivierung der Komplemente in salzfreien Medium. Berl. klin. Wochenschr. 16 p. 467-470. 17 p. 520-523. 19 p. 602-604.

33. Takenomata, U.:

1924. Beiträge zur Kenntnis des hämolytischen Komplements und seiner Komponenten. Zeitschr. f. Immunitätsf. 41 p. 431-458.

34. Thomson, Oluf, and Lecchly, W.:

1911. Ueber die Brandische Modification des Komplement - "Mittelstücks". Zeitschr. f. Immunitatsf. 11 p. 216-234.

35. Tsurusaki, H.:

1908. Zur Kenntnis der Komplexen Hämolysine. Biochemische Zeitschrift 10 p. 345-355.

BIOGRAPHY

Born September 8, 1894, in Colorado Springs, Colorado.

Attended a private grammar school and Cutler Academy, former preparatory school for Colorado College.

A. B. Vassar College - 1917.

Assistant in Bacteriology, Nurses Training Camp, Vassar College - Summer, 1918.

Assistant to Dr. C.-E. A. Winslow in the Department of Public Health, American Museum of Natural History, New York City, 1918-1919.

Attended Marietta College 1921-1922.

Entered the School of Hygiene and Public Health, Johns Hopkins University in the fall of 1922.